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# Stromal plasma cells expressing immunoglobulin G4 subclass in non-small cell lung cancer

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**Key words**

IgG4, plasma cell, lung, carcinoma, tissue microarray

**Running head**

IgG4+ plasma cells in lung cancer

## Abstract

Inflammatory cell infiltration in tumor stroma may represent the interaction between the tumor and the immune system. The significance of immunoglobulin (Ig)G4+ plasmacytic infiltration, however, is poorly understood. Here, we analyzed the number of stromal IgG4+ plasma cells and the IgG4/IgG ratio of plasma cells in 294 primary non-small cell lung cancers (NSCLC) using tissue microarray (TMA) and conventional surgical specimens. In TMA, 35 (12%) cases of NSCLC revealed more than 20 IgG4+ plasma cells per high-power field. In surgical specimens, most (97%) of those IgG4+ plasma cell-enriched cases showed obliterative phlebitis or arteritis, one of the key morphologic features of IgG4-related disease, within or at the periphery of the tumor. Clinically, none of the patients showed symptoms associated with IgG4-related systemic diseases. In patients with stage I squamous cell carcinoma, IgG4-enriched stroma was significantly associated with a favorable prognosis ($p = 0.04$). In conclusion, considerable IgG4+ plasma cell infiltration can be seen in a minority of cases of NSCLC, and might contribute to prognostic modulation of NSCLC.

## Introduction

Despite a number of recent studies focused on the tumor microenvironment, the role of plasma cells in cancer is not well understood [1]. The class and subclass of immunoglobulin (Ig) produced by plasma cells are defined primarily by their heavy chain constant domain sequences, which affect the function of the antibody [2]. IgG4, the least abundant subclass in the IgG class, shows weak or negligible binding ability to both C1q and Fcγ receptors compared with the other IgG subclasses, which leads to a limited capacity to activate the classical complement pathway [2,3]. Thus, until recently, it was believed that IgG4 plays only a limited role in immune activation [3]. As a consequence of the current familiarity of IgG4-related diseases (IgG4-RD) to pathologists and the widespread use of anti-IgG4 antibody for immunohistochemical staining, cases of carcinoma accompanied by severe IgG4+ plasma cell infiltration in patients with or without a medical history of IgG4-RD have been identified [4-9], although the prevalence and significance of this phenomenon remain unclear.

Differential diagnosis of IgG4-RD and cancers can be problematic, since IgG4-RD often forms tumefactive lesions and peritumoral desmoplastic tissue can mimic IgG4-RD when there is an abundance of IgG4+ plasma cells in a biopsy specimen. Some morphologic findings such as obliterative phlebitis are considered to be rather more specific to IgG4-RD than to cancer, but existing evidence supporting this idea

seems weak [10]. Serum IgG4 concentration is a helpful laboratory finding to differentiate IgG4-RD and cancer, but it is not always elevated in patients with IgG4-RD, while it can be elevated in cancer patients without IgG4-RD [4,8]. In addition, clinicians must keep in mind the possibility of synchronous cancer and IgG4-RD [11].

In this study, we identified cases of lung cancer with stromal IgG4+ plasma cell infiltration using tissue microarray (TMA). We analyzed the clinicopathologic characteristics of these cases to investigate the relevance of lung cancer and IgG4+ plasma cells.

## Materials and Methods

### TMA

Paraffin-embedded tumor blocks from 363 cases of lung cancer surgically resected from different patients in Kyoto University Hospital between 2001 and 2007 were selected from the electronic pathology files of Kyoto University Hospital for construction of TMA, with the patients' consent. TMAs were constructed by 2 of the authors (A.Y. and S.S.) basically using the approach described by Kononen et al. [12]. Briefly, the most morphologically representative region of the tumor was selected on a hematoxylin and eosin (H&E)-stained slide. Then, a tissue core of 2 mm in diameter was punched out from each donor tumor block using a thin-walled stainless steel

needle for TMA construction (Azumaya, Tokyo, Japan), and arrayed in a recipient paraffin block. Small cell carcinomas, recurrent carcinomas, carcinomas with neoadjuvant chemotherapy and cases with multiple carcinomas (both synchronous and metachronous) were excluded from this study, but were established in TMA for another study (n=45).

**Immunohistochemistry**

Immunostains against IgG4 and IgG using mouse anti-human IgG4:HRP (MCA2098P; dilution 1:200; AbD Serotec, Oxford, UK) and IgG (immunoglobulin G) (polyclonal; dilution 1:2 of prediluted product; Ventana, Tucson, AZ, USA) were performed on an autoimmunostainer (Ventana XT System Benchmark; Ventana Medical Systems).

**Evaluation of immunohistochemistry**

In TMA sections, we regarded tumors infiltrated by more than 20 IgG4+ plasma cells per high-power field (HPF) (x40 objective lens with field number 22) as IgG4+ cell-enriched cases, since this criterion is used for diagnosing IgG4-RD in lung biopsy specimens [10]. To calculate the IgG4/IgG ratio in each TMA section, the total numbers of IgG4+ and IgG+ plasma cells per core were counted manually under a

light microscope by M.F. and A.Y. For those IgG4+ cell-enriched cases with >25% IgG4/IgG ratio, immunohistochemistry for IgG4 and IgG was performed on the original surgical specimens to extract the cases fulfilling the quantitative criteria for IgG4-RD (>50/HPF of plasma cells with IgG4/IgG ratio >40%) [10]. In each case, IgG4 and IgG were counted in the region most highly stained for IgG4 in three HPFs to calculate the IgG4/IgG ratio.

**Histologic evaluation**

Histologic types of lung cancers were determined according to the 2004 WHO classification [13]. In adenocarcinomas, the predominant histologic subtype or variant in each case was identified according to the new lung adenocarcinoma classification proposed by the International Association for the Study of Lung Cancer, American Thoracic Society and European Respiratory Society (IASLC/ATS/ERS classification) [14], and it was also graded as well differentiated (G1), moderately differentiated (G2) and poorly differentiated (G3) based on the 2004 WHO classification by three of the authors (M.F., A.Y. and S.S.) [13]. Squamous cell carcinomas were not graded in this study, since the stage of the disease and the performance status at diagnosis remain the most powerful prognostic indicators, instead of the current grading system mainly based on the degree of keratinization [13]. IgG4+ cell-enriched cases were

evaluated by two of the authors (M.F. and A.Y.) for the presence of obliterative phlebitis/arteritis and increased number of eosinophils, which we defined as more than 50 eosinophils per 10 HPFs.

**Clinical information**

The electronic chart of Kyoto University Hospital, the electronic database of the Department of Thoracic Surgery of Kyoto University Hospital and the electronic pathology database of Kyoto University Hospital were used to search for patients' prognosis and medical history.

**Somatic *EGFR*, *KRAS* and *p53* mutations**

*EGFR*, *KRAS* and *p53* mutation analyses were carried out in selected cases by methods described previously [15,16].

**Statistical analysis**

GraphPad Prism (GraphPad Software, San Diego, CA, USA) and JMP Start Statistics (Statistical Discovery Software SAS Institute, Cary, NC, USA) were used for statistical analyses. Comparisons between two groups were performed with Fisher's exact test or Mann-Whitney's test to analyze categorical variables and continuous

variables, respectively. The Kaplan-Meier method was used to evaluate patients' prognosis and log-rank tests were used to compare the survival rates among groups. Logistic regression analysis was performed to examine the interaction of the multiple clinicopathological variables. Significance was defined as $p<0.05$.

## Results

TMA cores that were not completely enclosed on the sections or did not include viable tumor on the sections were excluded from this study (n=24). As a result, 294 primary non-small cell lung carcinomas (NSCLC) were extracted. The median follow-up period of patients was 1748 days (range: 7-3805).

The 294 lung cancers consisted of 233 adenocarcinomas (79.3%), 52 squamous cell carcinomas (17.7%) and 9 other NSCLC (3.1%) (6 large cell carcinomas, 2 sarcomatoid carcinomas and 1 adenosquamous carcinoma). According to the 7$^{th}$ UICC TNM classification [17], 215 cases (73.1%) were stage I, 37 cases (12.6%) were stage II, 31 cases (10.5%) were stage III and 11 cases (3.7%) were stage IV. Among the 233 adenocarcinomas, 60 cases (25.8%) were G1, 90 cases (38.6%) were G2 and 83 cases (35.6%) were G3. The median numbers of IgG4+ and IgG+ plasma cells in the TMA core were 0 (range: 0-570) and 134 (range: 0-2178),

respectively. None of the 294 patients had IgG4-RD in their medical history and serum IgG4 concentration was not measured in any of the patients.

In adenocarcinomas, mutation analysis of *EGFR*, *KRAS* and *p53* was performed in 213 cases, 213 cases and 129 cases, respectively, and the mutation was confirmed in 102 cases (47.9%), 23 cases (10.8%) and 31 cases (24.0%), respectively.

**Lung cancers with IgG4+ cell enrichment (n=35)**

Out of 294 cases, 35 cases (11.9%) were IgG4+ cell-enriched**,** including 17 adenocarcinomas (7.3% of all adenocarcinomas), 17 squamous cell carcinomas (32.7% of all squamous cell carcinomas) and 1 adenosquamous carcinoma. Among the 17 adenocarcinomas, 15 cases were G3 and 2 were G2. G1 adenocarcinoma was not observed.

The median number of original tumor slides observed per case was 3 (range: 1-9) and Victoria blue H&E stain and/or Elastica van Gieson stain was performed in all but 1 case. Fibrosis was evident in cancer stroma of all 35 cases, but none of them showed storiform pattern fibrosis. Obliterative phlebitis/arteritis was found in 34 cases (97.1%) associated with desmoplasia. An increased number of eosinophils was seen in 3 cases (8.6%). In all 35 cases, pathological change for which IgG4-RD was suspected was absent in non-neoplastic lung tissue surrounding the cancers.

The result of comparison between IgG4+ cell-enriched cases and the cases with ≤20 IgG4+/HPF in the TMA core is shown in Table 1. We also performed multivariate logistic regression analysis to determine which factors, among those showing statistical significance in univariate analysis, were more significantly associated with >20/HPF IgG4+ plasma cell infiltration (Table 2). This analysis revealed that squamous cell carcinoma and G3 adenocarcinoma were associated with >20/HPF IgG4+ plasma cell infiltration with high statistical significance ($p<0.0001$ and $p=0.0013$, respectively).

No statistical difference in recurrence rate or death rate was observed between the two groups (Table 1). In stage I carcinoma, however, none of the 19 IgG4+ cell-enriched cases (10 G2-G3 adenocarcinomas, 8 squamous cell carcinomas and 1 adenosquamous carcinoma) was associated with patient death, and only 1 squamous cell carcinoma and 1 adenocarcinoma recurred. In contrast, among 27 stage I squamous cell carcinomas with ≤20 IgG4+ plasma cells/HPF, 10 patients (37.0%) died of disease and 11 cases (40.7%) recurred. Among 110 stage I G2-3 adenocarcinomas with ≤20 IgG4+ plasma cells/HPF, 13 patients (11.8%) died of disease and 29 cases (26.4%) recurred. A significant difference of overall patient survival was observed in stage I squamous cell carcinoma ($p=0.0409$), but this was not the case for disease-free survival rate ($p=0.1053$) (Figure 1). No significant

difference in overall survival or disease-free survival rate was observed in stage I G2-3 adenocarcinoma (p=0.3280 and p=0.4407, respectively).

**Lung cancers with >50 IgG4+ plasma cells/HPF and >40% IgG4/IgG plasma cells in surgical specimens (n=6)**

Among 35 IgG4+ cell-enriched cases in the TMA core, 12 cases were associated with >25% IgG4/IgG ratio, and 6 of them showed >50 IgG4+ plasma cells/HPF and >40% IgG4/IgG ratio in the surgical specimens. The clinicopathological features of these 6 cases are summarized in Table 3. Histologic figures of selected cases are shown in Figure 2.

In comparison of these 6 cases and the other 29 IgG4+ cell-enriched cases, no statistically significant difference was obtained in any parameters shown in Table 1, except the IgG4/IgG ratio in the TMA core (median: 0.28 vs. 0.12, p=0.0062). Additionally, the numbers of cases with obliterative phlebitis/arteritis and increased number of eosinophils in these 2 groups did not differ significantly (83.3% vs. 100%, p=0.1714, and 16.7% vs. 6.9%, p=0.4417, respectively).

## Discussion

IgG4+ plasma cell infiltration in cancer and its relevance to IgG4-RD have been a

matter of debate in the field of practical medicine [4-9]. Since chronic inflammation is associated with a risk of cancer development, it could be hypothesized that IgG4-RD is a source of carcinoma [11,18]. In our series, however, the idea that the patients with IgG4+ cell-enriched tumor had lung IgG4-RD seems unlikely for two main reasons. Firstly, none of the IgG4+ cell-enriched cases showed histology of IgG4-RD in the non-neoplastic lung tissue, suggesting that the cancer is the factor causing the inflammation and not the other way round. Secondly, although IgG4-RD is a systemic disease and the affected patients commonly develop multi-visceral lesions or multiple lesions in the same organ at the time of diagnosis or during the follow-up [6,19], none of the patients in our series was clinically complicated by IgG4-RD at the time of diagnosis or during the follow-up of cancer.

Considering the favorable prognosis observed in IgG4-enriched stage I squamous cell carcinomas, IgG4 infiltration might cause prognostic modulation of NSCLC. At first, this result seemed to be inconsistent with the notable anti-immune function of IgG4, as seen in the transition of serum IgG4 level in immunotherapy [20]. Other previous reports, however, show that the functional role of IgG4 varies in different pathophysiological circumstances, and that IgG4 can be a pathogenic antibody in certain immunologic disorders [21-25]. Our data may suggest that IgG4 reacts against certain lung cancer antigens and damages tumor cells in spite of its poor complement-

and leucocyte-activating properties. By multivariate analysis, we found that squamous cell carcinomas and high-grade adenocarcinomas are more likely to be accompanied by intra-tumoral IgG4+ plasma cells; however, the reason for this tendency is unclear at present.

In stage I squamous cell carcinoma, intra-tumoral IgG4+ plasma cell infiltration may be a better prognostic factor than conventional histological grade because the histological grade of lung squamous cell carcinoma is not clearly related to its prognosis [13]. IgG4+ plasma cells might have a cancer-inhibiting role in adenocarcinomas as well, but our data did not show statistical significance. In adenocarcinomas, high-grade histology or non-EGFR gene mutation may be a more powerful prognostic factor than IgG4+ cell infiltration [15,16,26].

A common histological finding of IgG4-RD is sclerosing fibrosis arranged at least focally in a storiform pattern accompanied by marked IgG4+ plasma cell infiltration, usually associated with obliterative phlebitis; however, there is variability in the findings in certain organs [10]. In lung IgG4-RD, storiform-type fibrosis is uncommon; thus, histological findings tend to overlap with other pulmonary fibroinflammatory conditions. In the pericancerous fibrous stroma of our IgG4+ cell-enriched cancers, even obliterative phlebitis/arteritis, the histological finding of which is believed to be quite specific to lung IgG4-RD, was observed in most of the cases. Pathologists

should be aware of this fact when diagnosing lung IgG4-RD on biopsy specimens.

In conclusion, a non-negligible minority of cases of squamous cell carcinoma and high-grade adenocarcinoma of the lung showed IgG4+ plasma cell infiltration in the stroma. Obliterative vascular changes were commonly seen in those cases without clinical evidence of IgG4-RD. Since IgG4+ cell-enriched cases were associated with favorable prognosis in stage I squamous cell carcinomas, IgG4 infiltration may be part of the anti-cancer immune response.

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## Table legends

Table 1. Comparison of lung cancers with and without >20 IgG4+ plasma cells/HPF in the tissue microarray (TMA) core (n=294).

Table 2. Logistic regression analysis for estimation of significant factors related to >20/HPF intra-tumoral IgG4+ plasma cell infiltration.

Table 3. Clinicopathological characteristics of 6 lung cancers that fulfilled the histological criteria of lung IgG4-RD (IgG4/IgG ratio >40% and >50/HPF IgG4+ plasma cells in surgical specimen).

Table 1

| | Number of IgG4+ plasma cells/TMA core | | p |
|---|---|---|---|
| | >20/HPF | ≤20/HPF | |
| No. of cases | 35 (11.9%) | 259 (88.1%) | |
| Median of IgG4/IgG ratio in TMA core (range) | 0.13 (0.021-0.47) | 0 (0-0.75) | <0.0001* |
| Male | 30 (85.7%) | 144 (55.6%) | 0.0005* |
| Median age (range) | 72 (41-88) | 67 (23-94) | 0.1783 |
| Never smokers | 4 (11.4%) | 107 (41.3%) | 0.0004* |
| Preoperative history of cancer | 7 (20.0%) | 48 (18.5%) | 0.8189 |
| Preoperative history of asthma | 4 (11.4%) | 8 (3.1%) | 0.0417* |
| Preoperative history of collagen vascular disease | 1 (2.8%) | 9 (3.5%) | 1 |
| Preoperative history of interstitial pneumonia | 1 (2.9%) | 15 (5.8%) | 0.7032 |
| Squamous cell carcinomas | 17 (48.6%) | 35 (13.5%) | <0.0001* |
| G3 adenocarcinomas | 15 (42.9%) | 68 (26.3%) | 0.0468* |
| Gene mutation in adenocarcinomas | EGFR 3/14 (21.4%) | EGFR 99/199 (49.0%) | 0.0524 |
| | KRAS 1/14 (7.1%) | KRAS 22/199 (11.1%) | 1 |
| | p53 5/7 (71.4%) | p53 26/122 (21.3%) | 0.0088* |
| Stage I cancers | 19 (54.3%) | 196 (75.7%) | 0.0134* |
| Recurrence rate (Recurrence rate of stage I cancers) | 34.3% (10.5%) | 32.8% (20.9%) | 0.8502 (0.3773) |
| Death rate (Death rate of stage I cancers) | 20.0% (0%) | 22.8% (12.2%) | 0.8111 (0.1395) |

* Statistically significant.

Table 2

| Variable | Odds ratio | 95% confidence interval | p |
|---|---|---|---|
| Sex (Male/Female) | 1.8285 | 0.5631-6.9714 | 0.3392 |
| Smoking history (Never/Current or Former) | 0.6118 | 0.1400-2.2837 | 0.4829 |
| Preoperative history of asthma (present/absent) | 2.7665 | 0.6253-11.2757 | 0.1581 |
| Squamous cell carcinoma | 15.4307 | 4.5863-71.6024 | <0.0001* |
| G3 adenocarcinoma | 8.5662 | 2.5946-38.8643 | 0.0013* |
| Cancer stage (Stage I/Stage II-IV) | 0.6655 | 0.2968-1.4987 | 0.3215 |

* Statistically significant.

Table 3

| Case No. | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Sex | Male | Male | Male | Male | Male | Male |
| Age at surgery | 59 | 61 | 61 | 65 | 72 | 75 |
| Smoking status | Current | Former | Former | Former | Never | Current |
| Past medical history | None | Hypopharyngeal squamous cell carcinoma | None | Asthma, Hepatitis C | Diabetes mellitus, Arrhythmia | Colon carcinoma, Renal carcinoma (histology not available) |
| Site | Right upper lobe | Left upper lobe | Right lower lobe | Right upper lobe | Left upper lobe | Left lower lobe |
| Stage | 1B (T2aN0M0) | 1A (T1aN0M0) | 1A (T1bN0M0) | 1B (T2aN0M0) | 2A (T1bN1M0) | 1A (T1aN0M0) |
| Histology | Adenosquamous carcinoma | Adenocarcinoma, solid predominant, G3 | Squamous cell carcinoma | Squamous cell carcinoma | Adenocarcinoma, micropapillary predominant, G3 | Squamous cell carcinoma |
| Obliterative phlebitis/ arteritis | Present | Present | Absent | Present | Present | Present |
| Eosinophilic infiltration >50/10 HPF | Absent | Absent | Absent | Present | Absent | Absent |
| IgG4/IgG ratio in TMA core (%) | 206/820 (25.1) | 570/1204 (47.3) | 172/625 (27.5) | 258/1012 (25.5) | 216/648 (33.3) | 348/1211 (28.7) |
| IgG4/IgG ratio in surgical specimen (%) | 51.7/103.7 (49.9) | 80.0/137.0 (58.4) | 67.7/146.7 (46.1) | 53.3/125.3 (42.5) | 69.7/170.7 (40.8) | 50.6/105.3 (48.1) |
| EGFR | Not done | Wild | Wild | Wild | Mutated | Wild |
| KRAS | Not done | Mutated | Wild | Wild | Wild | Wild |
| p53 | Not done | Not done | Mutated | Wild | Not done | Wild |
| Recurrence of lung carcinoma | Absent | Absent | Absent | Absent | Present | Absent |
| Outcome | Alive | Alive | Alive | Alive | Died of disease | Alive |
| Follow-up period (days) | 3480 | 1666 | 2747 | 2002 | 376 | 1468 |

## Figure legends

Figure 1 Kaplan-Meier survival curves of 35 patients with stage I squamous cell carcinomas separated by the number of IgG4+ plasma cells/HPF in the tissue microarray core (>20/HPF vs. ≤20/HPF). (A) Overall survival curves (log-rank test, p=0.0409), and (B) disease-free survival curves (log-rank test, p=0.1053).

Figure 2 Histology of lung cancers fulfilling the histological criteria for lung IgG4-RD, (A)-(E) case 2 and (F)-(H) case 4.

(A) Case 2, solid predominant G3 adenocarcinoma (×20).

(B) Higher-magnification image of boxed area in (A) (×100).

(C) Intratumoral obliterative phlebitis (×100).

(D) >50/HPF IgG4+ plasma cells in the cancer stroma (×200).

(E) IgG immunostain at the same area as in (D), with IgG4/IgG ratio >40% (×200).

(F) Case 4, squamous cell carcinoma (×40).

(G), (H) Higher magnification of the areas demarcated by boxes in (F); obliterative vasculitis and eosinophilic infiltration are shown, respectively (×200 and ×400).

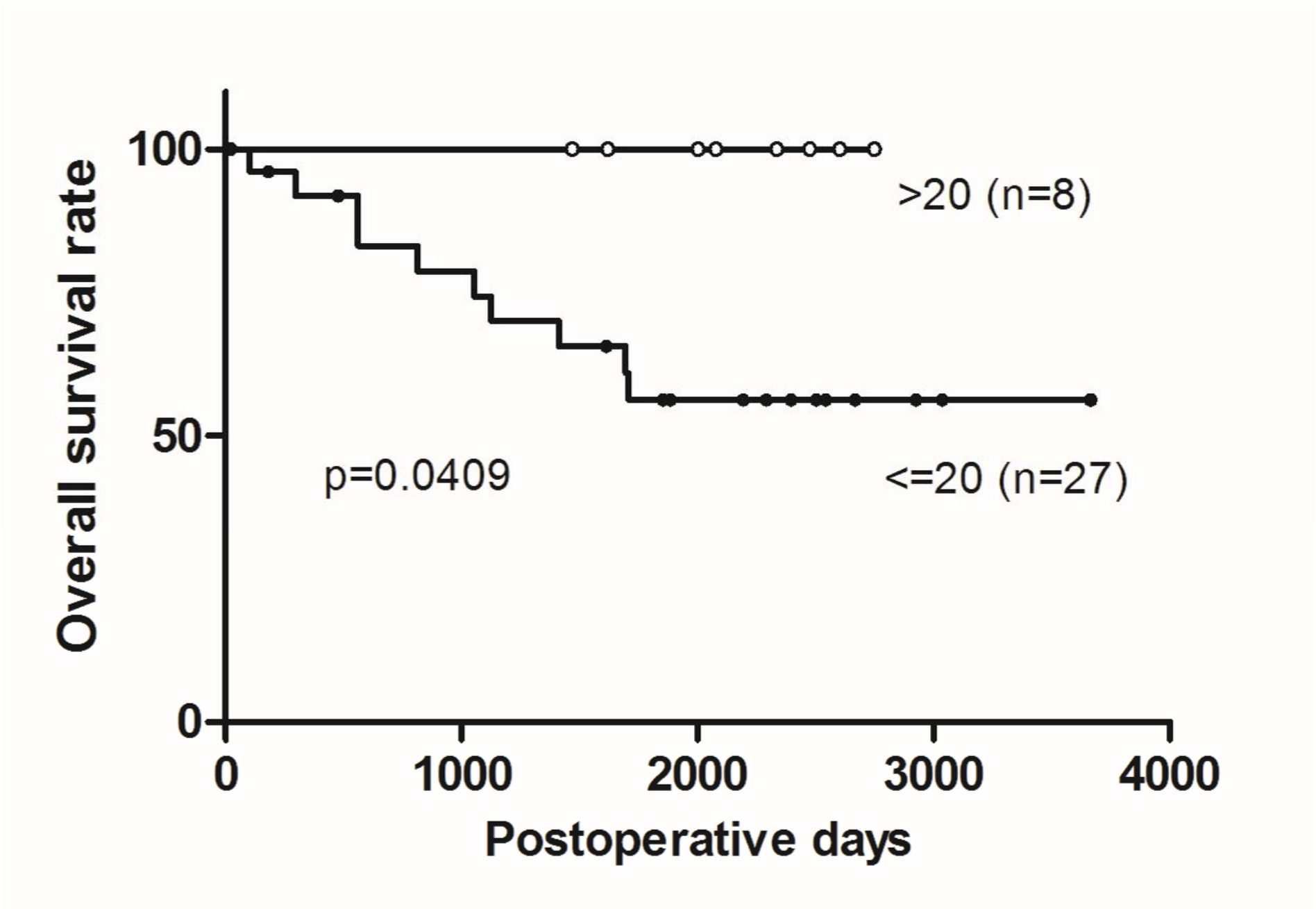
Overall survival rate
100
50
0
0
1000
2000
3000
4000
Postoperative days
>20 (n=8)
<=20 (n=27)
p=0.0409

Figure 1(A)

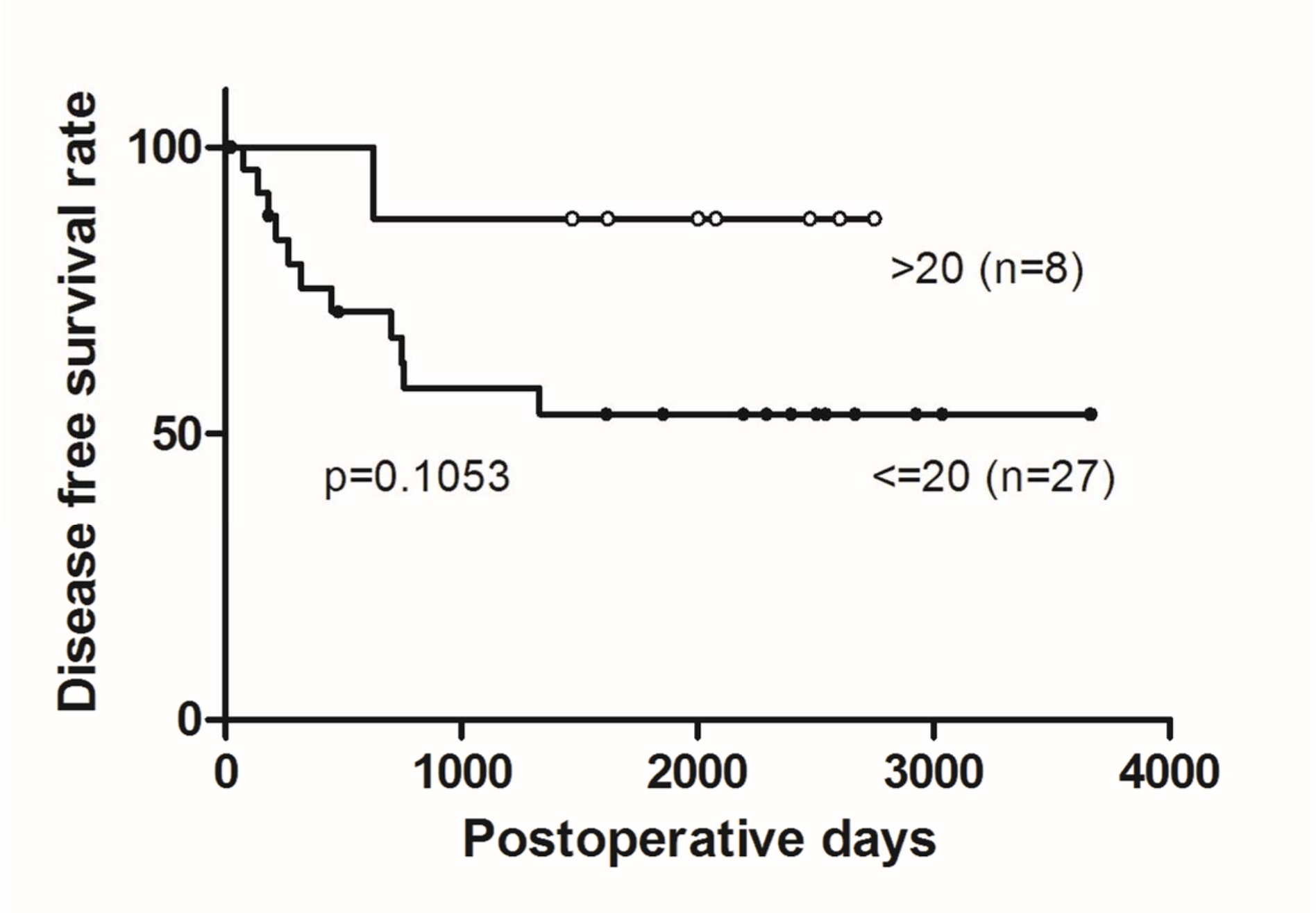
Disease free survival rate
100
50
0
0
1000
2000
3000
4000
Postoperative days
>20 (n=8)
<=20 (n=27)
p=0.1053

Figure 1(B)

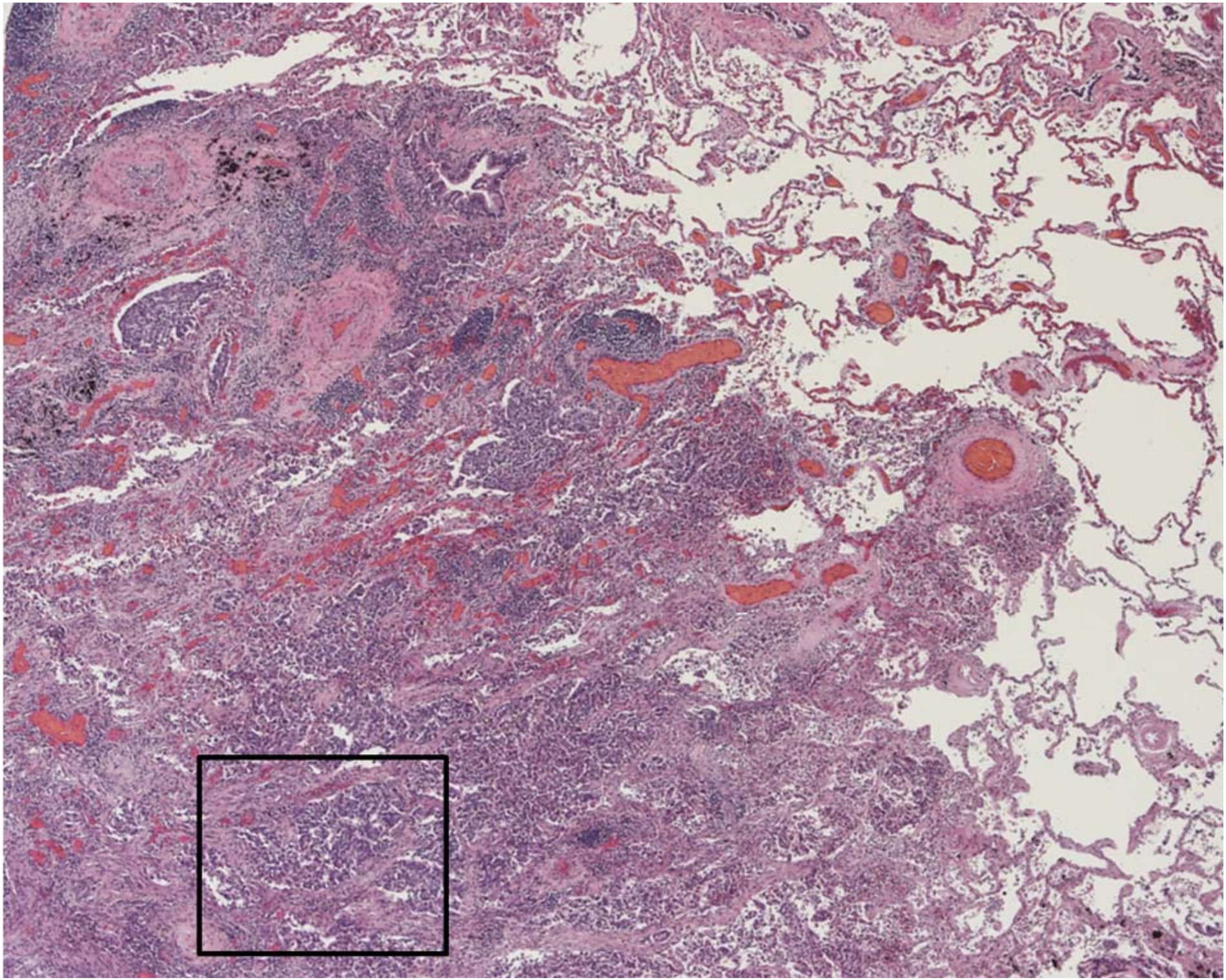

Figure 2(A)

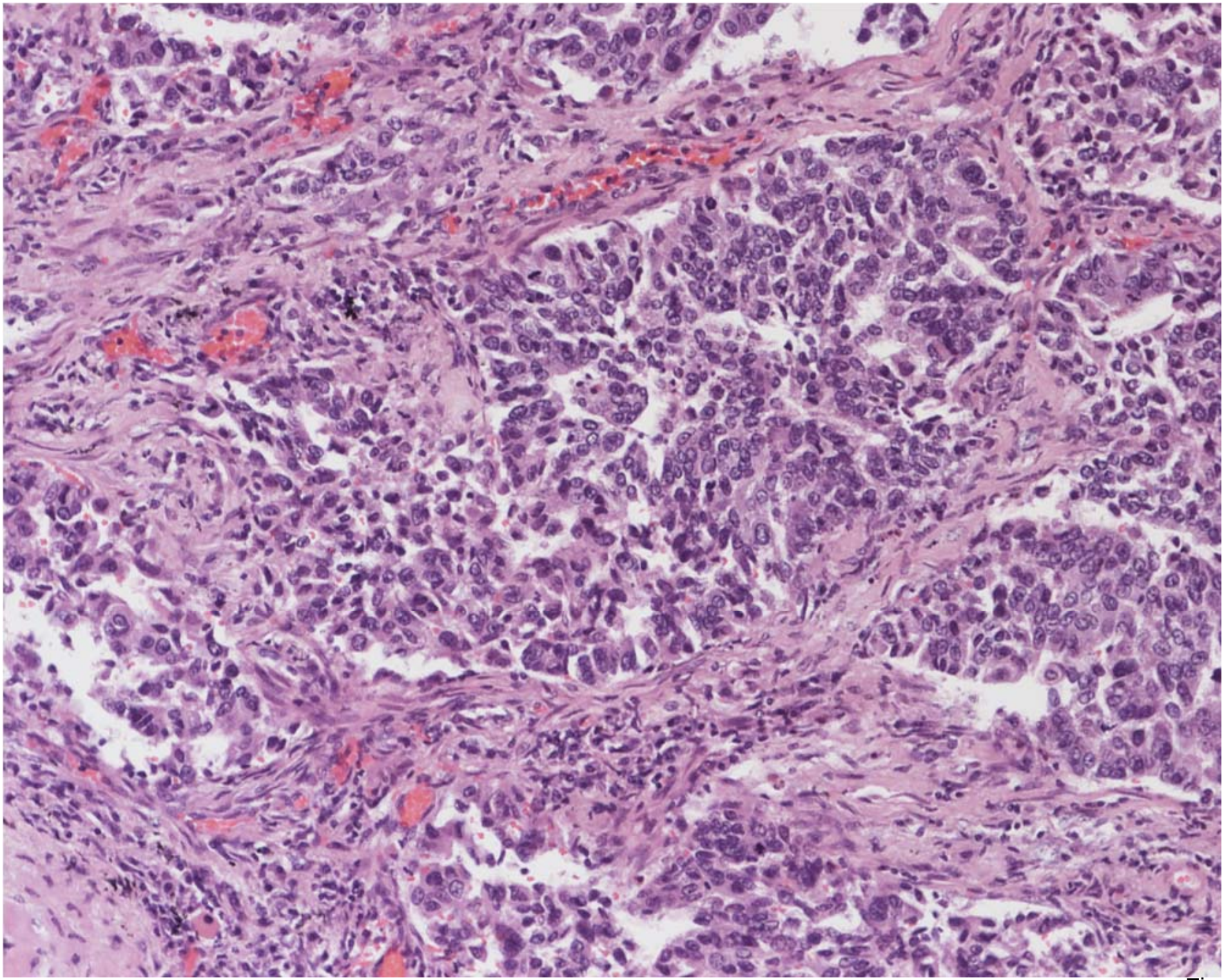

Figure 2(B)

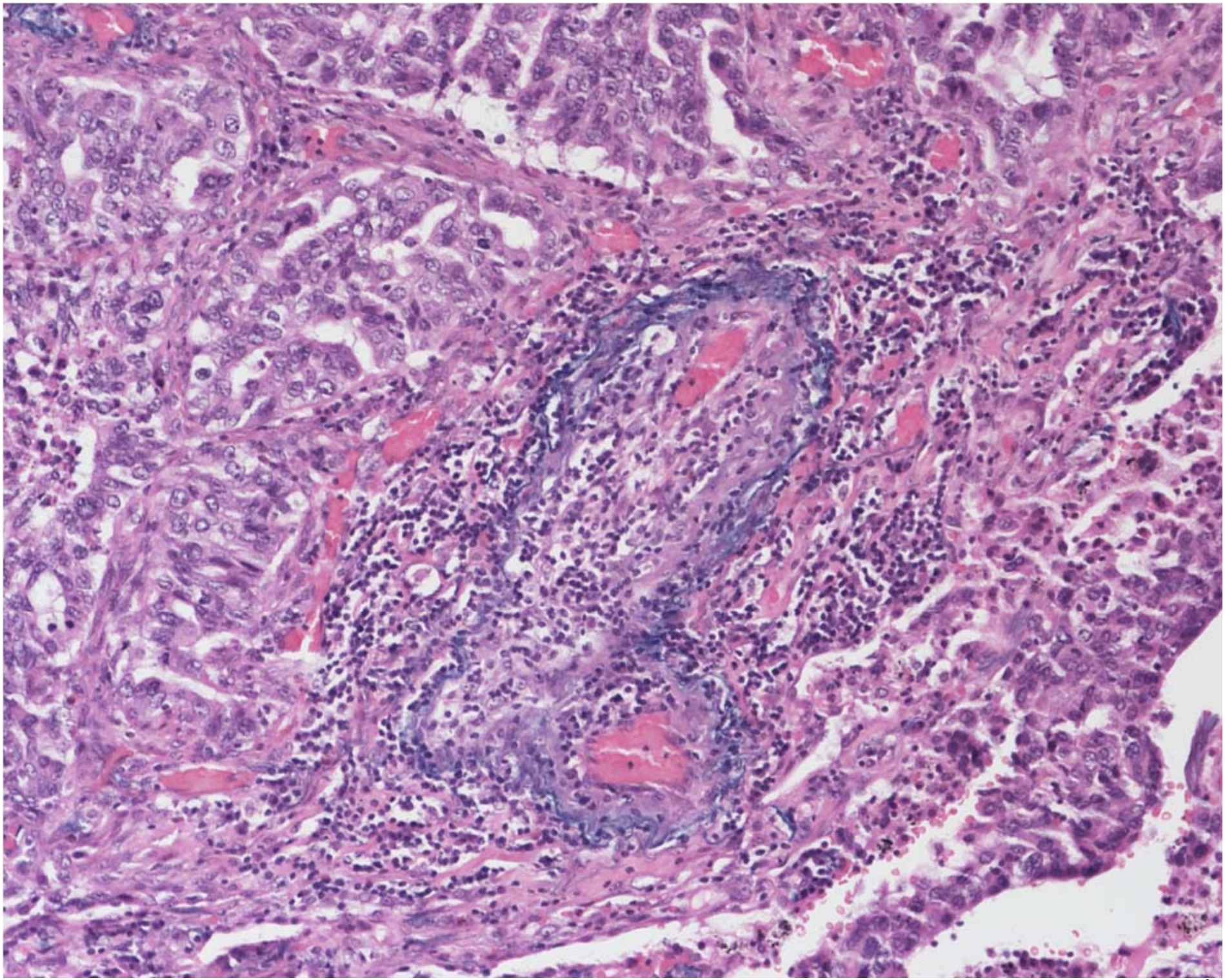

Figure 2(C)

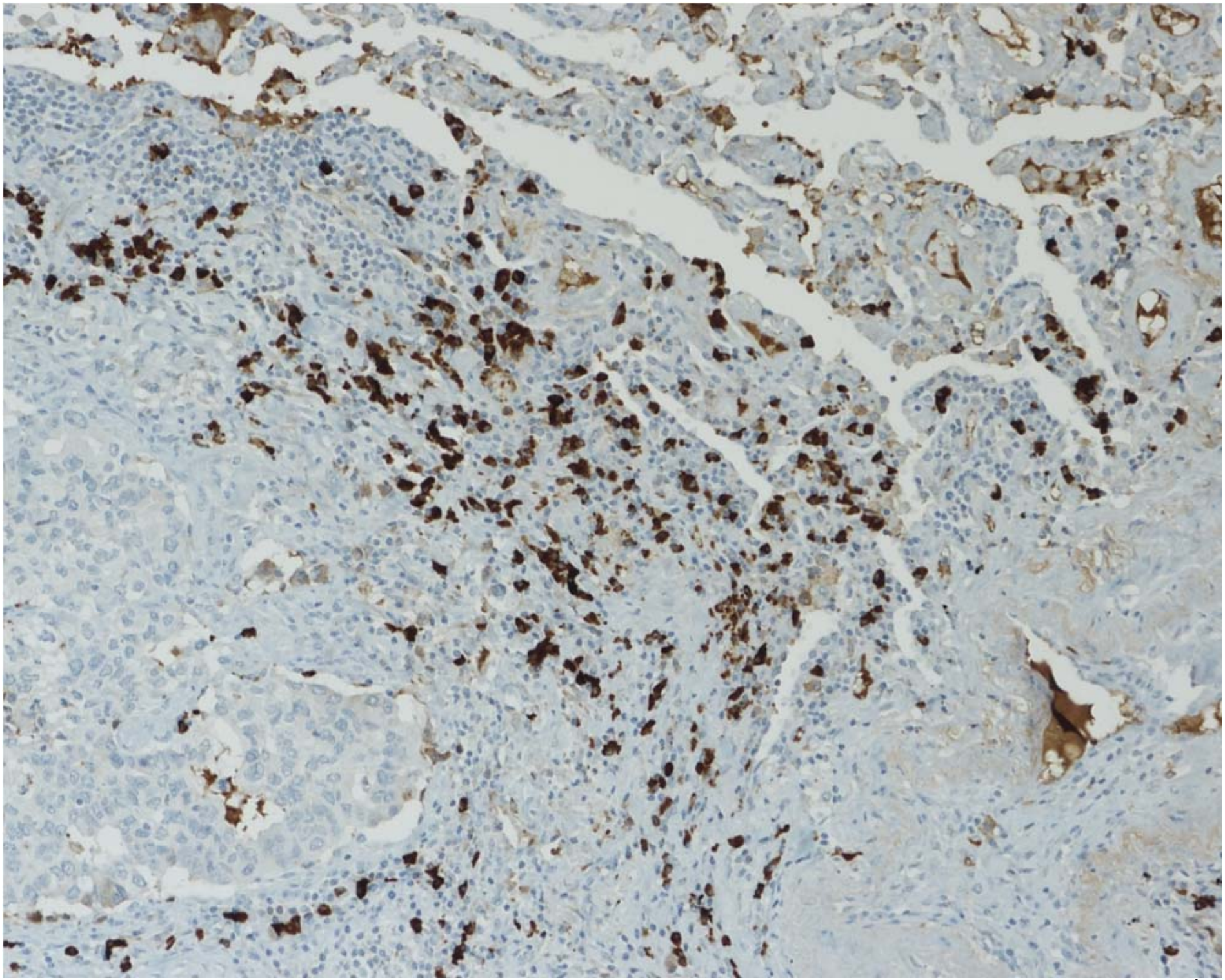

Figure 2(D)

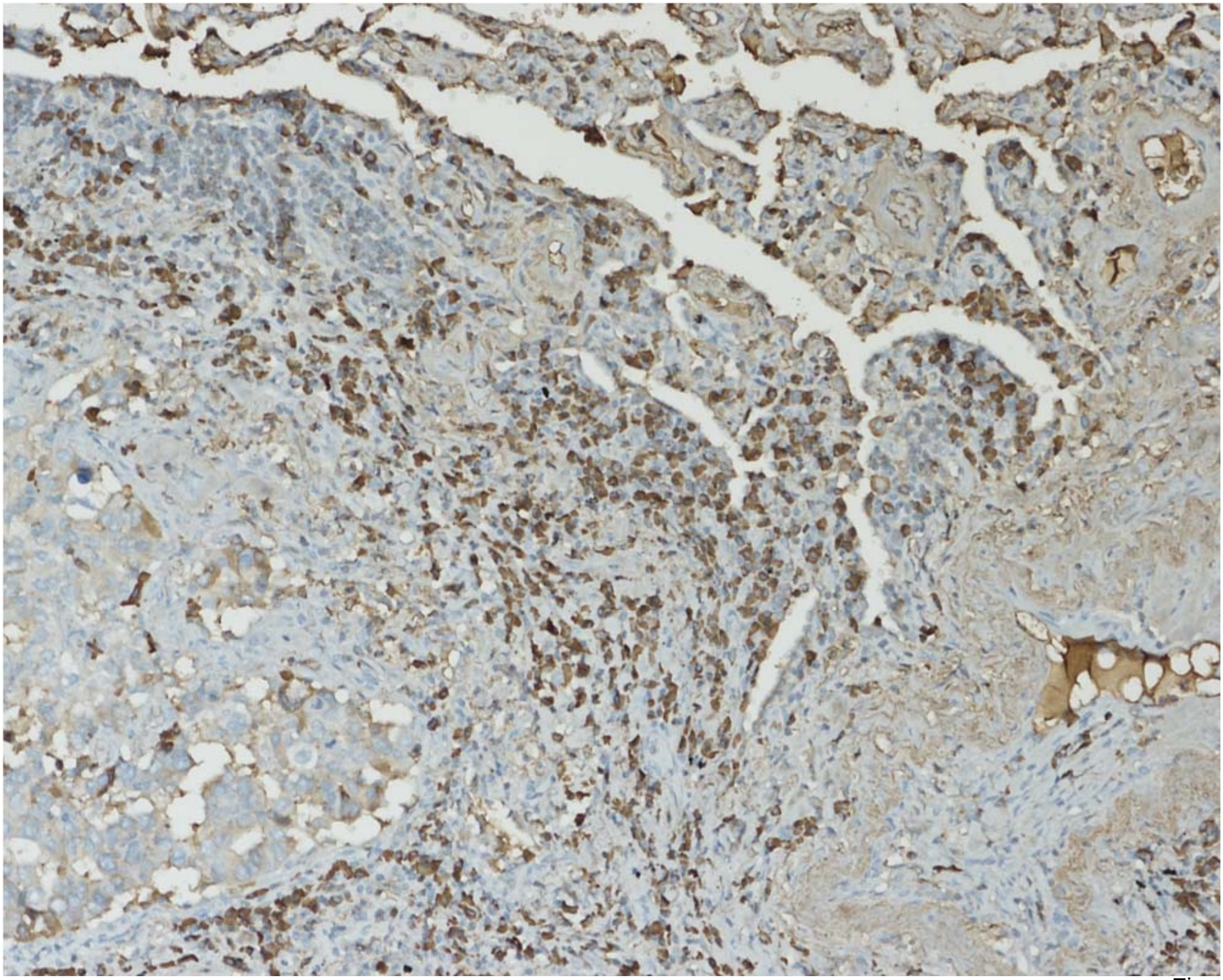

Figure 2(E)

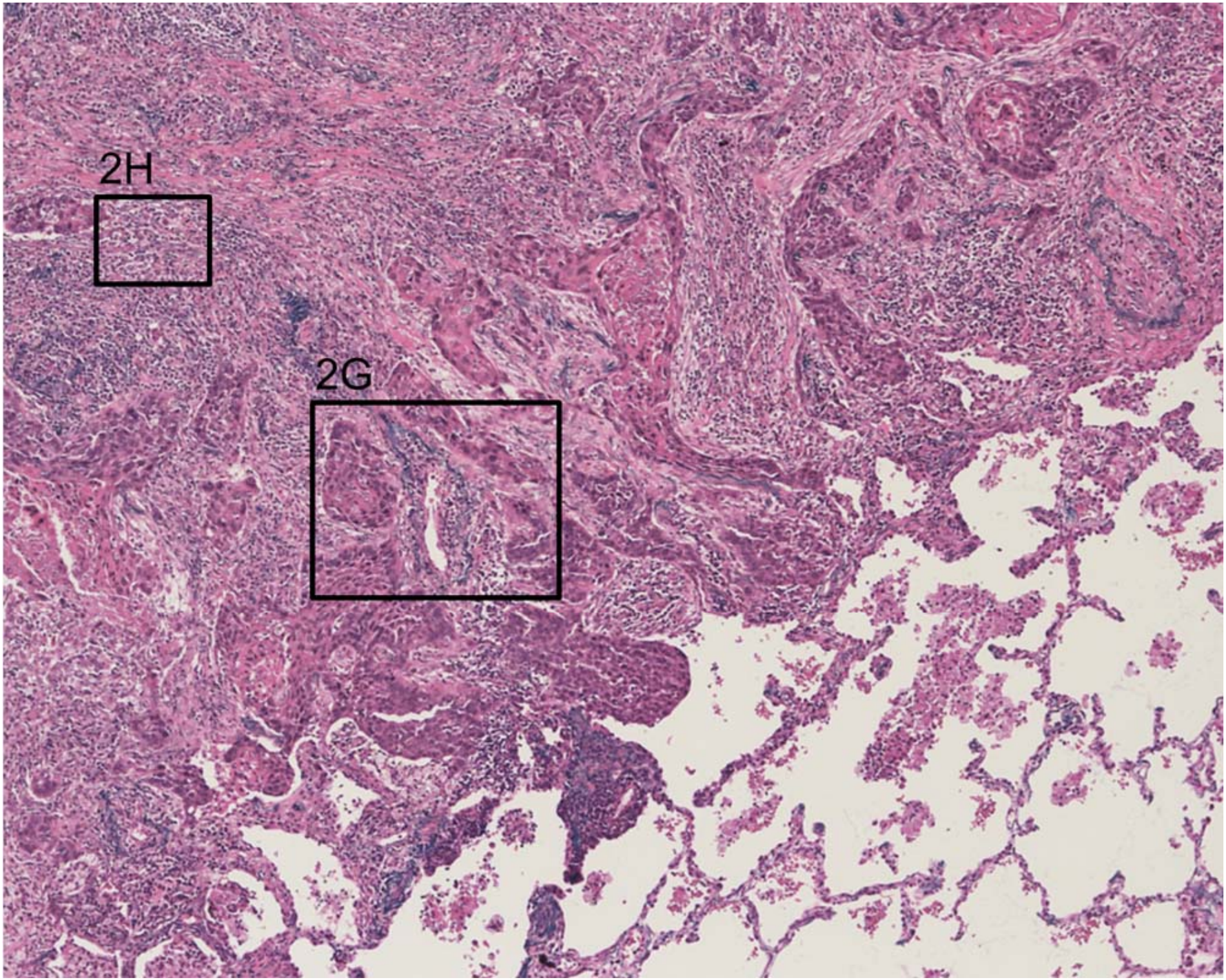


Figure 2(F)

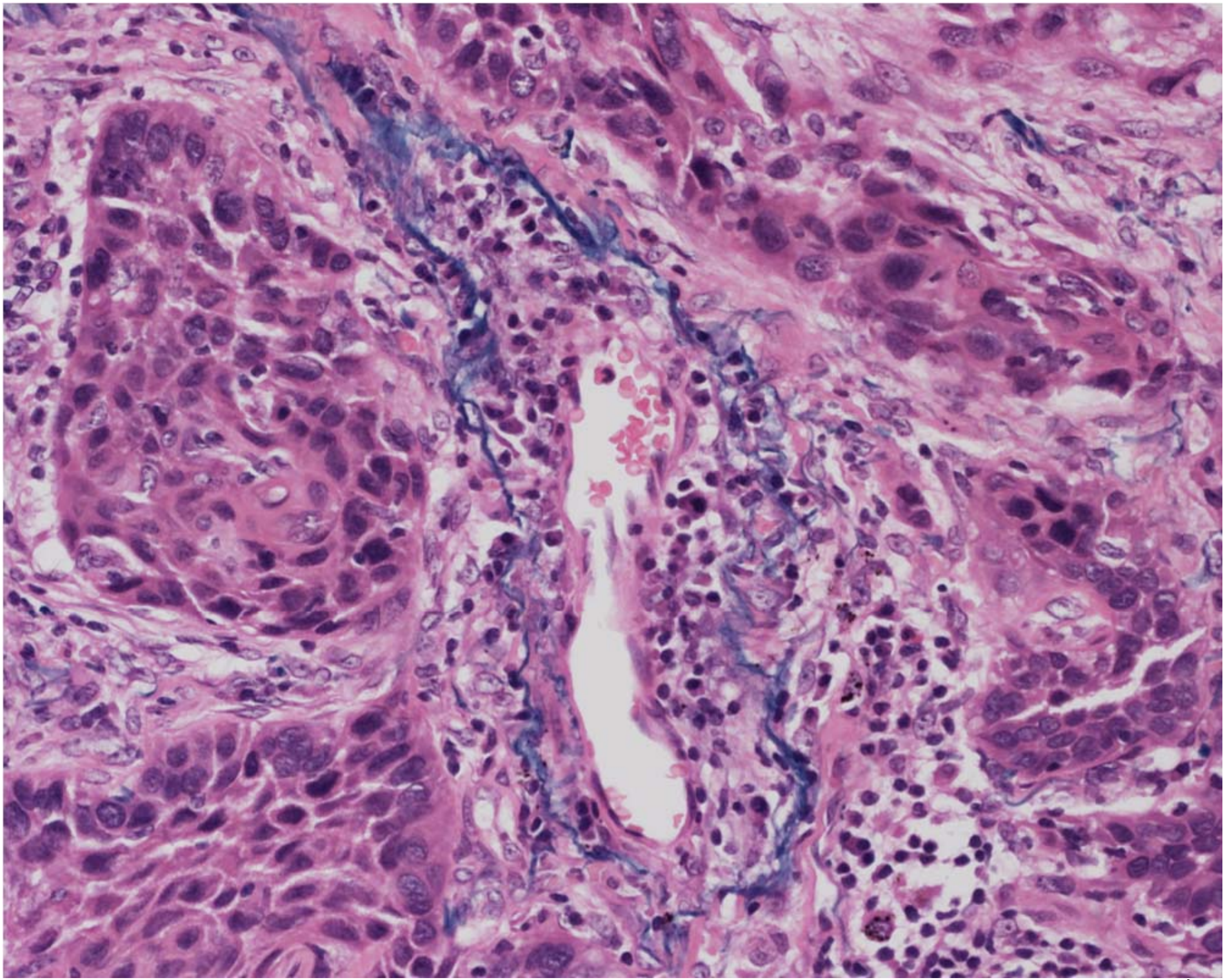

Figure 2(G)

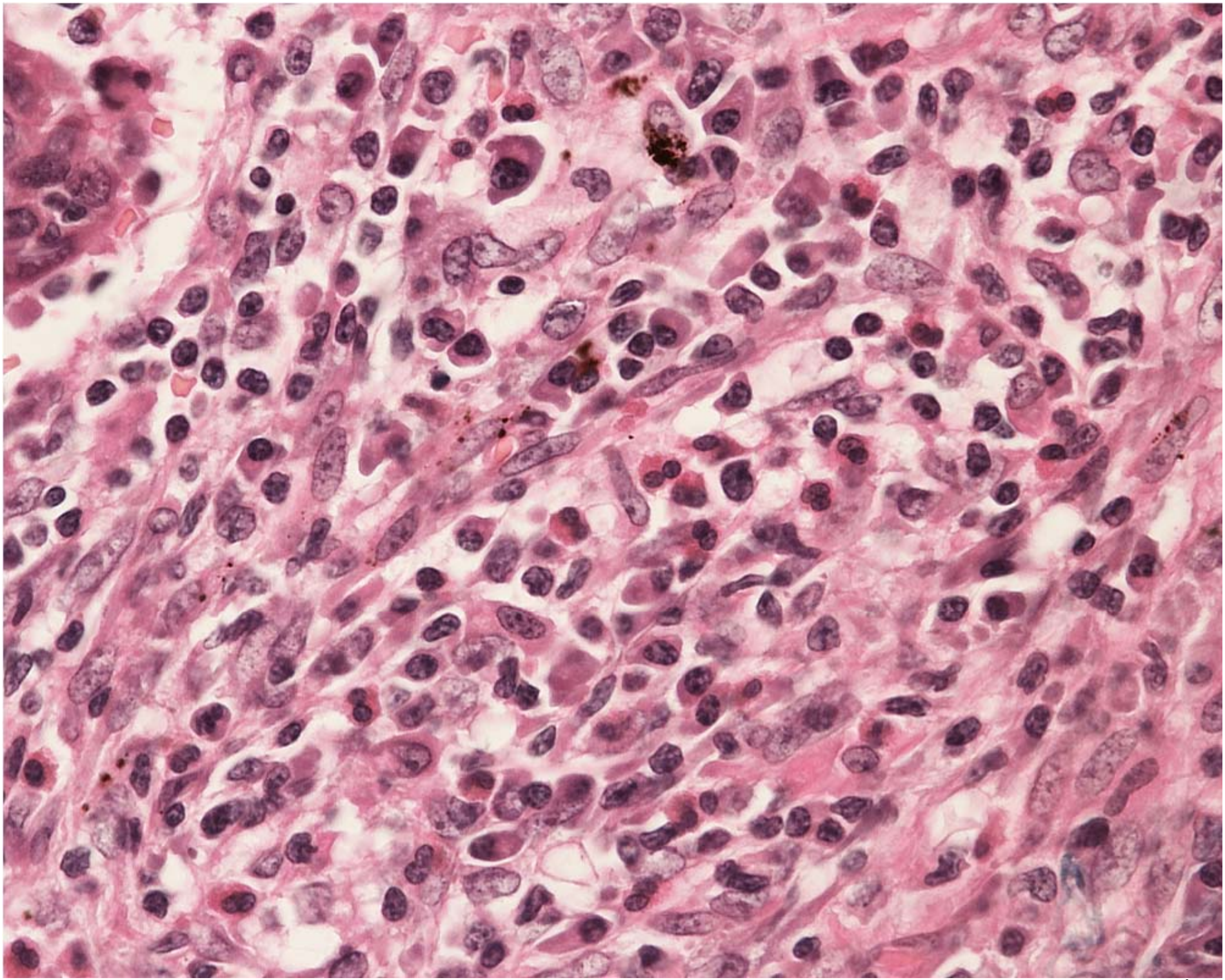

Figure 2(H)